A new pathogenicity island carrying an allelic variant of the Subtilase cytotoxin is common among Shiga toxin producing *Escherichia coli* of human and ovine origin

V. Michelacci[1,2], R. Tozzoli[1], A. Caprioli[1], R. Martínez[3], F. Scheutz[4], L. Grande[1], S. Sánchez[5] and S. Morabito[1]

1) *European Reference Laboratory for Escherichia coli, Istituto Superiore di Sanità, Dipartimento di Sanità Pubblica Veterinaria e Sicurezza Alimentare, Rome, Italy,* 2) *Department of Biology, University 'Roma Tre', Roma, Italy,* 3) *Patologia Infecciosa y Epidemiologia, Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad de Extremadura, Cáceres, Spain,* 4) *WHO Collaborating Centre for Reference and Research on Escherichia and Klebsiella, Department of Microbiology and Infection Control, Statens Serum Institut, Copenhagen, Denmark and* 5) *Laboratorio de Enterobacterias, Servicio de Bacteriologia, Centro Nacional de Microbiologia, Instituto de Salud Carlos III, Madrid, Spain*

Abstract

Subtilase (SubAB) is a cytotoxin elaborated by some Shiga Toxin (Stx)-producing *Escherichia coli* (STEC) strains usually lacking the locus of enterocyte effacement (LEE). Two variants of SubAB coding genes have been described: $subAB_1$, located on the plasmid of the STEC O113 98NK2 strain, and $subAB_2$, located on a pathogenicity island (PAI) together with the *tia* gene, encoding an invasion determinant described in enterotoxigenic *E. coli*. In the present study, we determined the entire nucleotide sequence of the PAI containing the $subAB_2$ operon, termed Subtilase-Encoding PAI (SE-PAI), and identified its integration site in the *pheV* tRNA locus. In addition, a PCR strategy for discriminating the two *subAB* allelic variants was developed and used to investigate their presence in *E. coli* strains belonging to different pathotypes and in a large collection of LEE-negative STEC of human and ovine origin. The results confirmed that *subAB* genes are carried predominantly by STEC and showed their presence in 72% and 86% of the LEE-negative strains from human cases of diarrhoea and from healthy sheep respectively. Most of the *subAB*-positive strains (98%) identified possessed the $subAB_2$ allelic variant and were also positive for *tia*, suggesting the presence of SE-PAI. Altogether, our observations indicate that $subAB_2$ is the prevalent SubAB-coding operon in LEE-negative STEC circulating in European countries, and that sheep may represent an important reservoir for human infections with these strains. Further studies are needed to assess the role of *tia* and/or other genes carried by SE-PAI in the colonization of the host intestinal mucosa.

Keywords: STEC, pathogenicity island, subtilase cytotoxin, diarrhoea, small ruminants, PCR
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Corresponding author: S. Morabito, Dipartimento di Sanità Pubblica Veterinaria e Sicurezza Alimentare, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy
E-mail: stefano.morabito@iss.it

Introduction

Subtilase (SubAB) is an AB_5 toxin produced by certain *Escherichia coli* strains associated with human disease [1]. SubAB is composed of a 35 kDa A subunit displaying a subtilase-like serine protease activity and five 13 kDa B subunits forming a pentamer, which mediates the binding to specific receptors on the host cell surface [1]. Following internalization in cultured cells, SubAB is delivered to the endoplasmic reticulum (ER) [2], where it has been demonstrated to cleave the chaperone BiP [3], causing the RNA-dependent protein kinase-like ER kinase activation and the transient inhibition of protein synthesis, resulting in the induction of the apoptotic signalling pathways [4–7].

SubAB has so far been identified almost exclusively in Shiga toxin (Stx)-producing *E. coli* (STEC), and in particular in strains that do not possess the locus for enterocyte effacement (LEE)

[8–18]. The LEE is a pathogenicity island (PAI) governing the attaching and effacing mechanism of intestinal adhesion [19], and represents a common feature of STEC strains associated with severe human disease. It has been hypothesized that the SubAB may contribute to the pathogenesis of STEC-associated human disease by playing a synergistic role with Stx [2]. As a matter of fact, SubAB has been shown to induce, in a mouse model, the typical haemolytic uraemic syndrome (HUS)-associated features caused by Stx, such as extensive microvascular damage, and thrombosis and necrosis in the brain, kidneys and liver [20].

The prototype SubAB-positive STEC strain 98NK2, belonging to serotype O113:H21 and isolated from an outbreak of HUS in South Australia [1], carries the subtilase-coding operon (*subAB*) on a large virulence plasmid designated as pO113, which also carries the *saa* gene, encoding an autoagglutinating adhesin possibly involved in the colonization of the host intestinal mucosa [21].

Recently, we reported the production of SubAB by two Stx-negative *E. coli* strains (ED 32 and ED 591), isolated from two unrelated cases of uncomplicated diarrhoea in Italy [22]. Genetic analyses showed that the nucleotidic sequences of the *subA* and *subB* genes were identical in the two strains and 90% similar to those of the corresponding genes present in the pO113 plasmid of strain 98NK2 [22]. Strains ED 32 and ED 591 were both LEE-negative and did not react in a *saa*-specific PCR assay. Moreover, differently from strain 98NK2, they harboured the *subAB* genes in the chromosome and next to another gene, *tia*, encoding an invasion factor previously described in enterotoxigenic *E. coli* (ETEC) [23]. An identical chromosomal region carrying *subAB* and *tia* was identified in the chromosome of other *subAB*-positive STEC strains [22], suggesting the existence of a putative pathogenicity island (PAI) vehiculating the *subAB* and *tia* virulence genes.

The presence of *subAB* genes among *E. coli* strains of human or animal origin has been investigated in several studies [8–18]. However, most of these studies did not involve the use of tools capable of distinguishing between the two allelic variants of the *subAB* gene. As an exception, a recent investigation conducted on STEC strains isolated from cattle, sheep and goats [14] reported a different distribution of the two *subAB* variants in the different animal species, with the $subAB_{98NK2}$, named by the authors $subAB_1$, associated with bovine strains, and $subAB_{ED32}$, termed $subAB_2$, more frequent among strains from small ruminants [14].

In the present work, we investigated the presence of the two allelic variants of the *SubAB* gene in human *E. coli* strains belonging to different pathotypes and in a large collection of LEE-negative STEC of human and ovine origin. Moreover, we determined the entire nucleotidic sequence of the putative PAI containing the $subAB_2$ operon in the prototype *E. coli* strain ED 32 and describe its gene content and insertion site.

Materials and Methods

Bacterial strains

The prototype *E. coli* strain ED 32, containing the $subAB_2$ allelic variant, is part of the culture collection of the Istituto Superiore di Sanità and has been previously described [22]. The panel of human strains investigated included 177 STEC strains belonging to 10 different serogroups and displaying different combinations of *stx*-coding genes, 26 enteropathogenic *E. coli* (EPEC), 32 enterotoxigenic *E. coli* (ETEC), 20 enteroaggregative *E. coli* (EAEC), 13 enteroinvasive *E. coli* (EIEC), all isolated from cases of diarrhoea, and one strain isolated from a urinary tract infection (uropathogenic *E. coli*, UPEC).

The diarrhoeagenic *E. coli* used in this study have been classified based on the presence of the virulence genes described to be associated to the different pathotypes in the literature. EPEC pathotype was identified by the presence of the intimin-coding *eae* gene, detected together with the plasmid-associated marker EAF for identifying typical EPEC. STEC were recognized by the presence of the *stx1* and *stx2* genes. The invasion plasmid antigen-coding gene *ipaH* and the enteroaggregative *E. coli* antiaggregation protein transporter gene *aat* (previously reported as CVD 432) were considered markers for EIEC and EAEC pathotypes respectively. Finally, ETEC strains were identified by the presence of the heat-stable and the heat-labile enterotoxin-coding genes (*est* and *elt*, respectively). All the virulence genes were amplified as previously described [22]. The PCR assay for the gene encoding the EAF determinant has been described in [24]. The only human UPEC was isolated from a patient suffering from urinary tract infection. Serotyping, including both O and H antigen identification, was performed by the WHO Collaborating Centre for Reference and Research on *Escherichia* and *Klebsiella* at the Statens Serum Institut (Copenhagen, Denmark).

All the STEC strains selected for this study lacked the *eae* gene, considered as a hallmark for the presence of the LEE locus. All the *E. coli* strains of human origin included in the study are part of the Statens Serum Institut (Copenhagen, Denmark) culture collection.

One hundred and twenty-three LEE-negative STEC strains isolated from sheep were included in the study. The strains had been isolated from healthy animals in Spain during a previous longitudinal study involving 12 different sampling visits (one sampling/month) at four different farms. All the animal